A Decaepitope Polypeptide Primes for Multiple CD8$^+$ IFN-γ and Th Lymphocyte Responses: Evaluation of Multiepitope Polypeptides as a Mode for Vaccine Delivery[1]

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Proteins are generally regarded as ineffective immunogens for CTL responses. We synthesized a 100-mer decaepitope polypeptide and tested its capacity to induce multiple CD8$^+$ IFN-γ and Th lymphocyte (HTL) responses in HLA transgenic mice. Following a single immunization in the absence of adjuvant, significant IFN-γ in vitro recall responses were detected for all epitopes included in the construct (six A2.1-, three A11-restricted CTL epitopes, and one universal HTL epitope). Immunization with truncated forms of the decaepitope polypeptide was used to demonstrate that optimal immunogenicity was associated with a size of at least 30–40 residues (3–4 epitopes). Solubility analyses of the truncated constructs were used to identify a correlation between immunogenicity for IFN-γ responses and the propensity of these constructs to form particulate aggregates. Although the decaepitope polypeptide and a pool of epitopes emulsified in IFA elicited similar levels of CD8$^+$ responses using fresh splenocytes, we found that the decaepitope polypeptide more effectively primed for in vitro recall CD8$^+$ T cell responses. Finally, immunogenicity comparisons were also made between the decaepitope polypeptide and a corresponding gene encoding the same polypeptide delivered by naked DNA immunization. Although naked DNA immunization induced somewhat greater direct ex vivo and in vitro recall responses 2 wk after a single immunization, only the polypeptide induced significant in vitro recall responses 6 wk following the priming immunization. These studies support further evaluation of multiepitope polypeptide vaccines for induction of CD8$^+$ IFN-γ and HTL responses. *The Journal of Immunology,* **2002, 168: 6189–6198.**

Vaccines based on killed or inactivated pathogens, recombinant or purified proteins, are generally effective in inducing Th lymphocytes (HTL)[3] and Ab responses, but are generally ineffective at induction of CTL responses (1–3). This limitation presents a serious drawback for vaccines and immunotherapeutic regimens targeting diseases for which induction of CTL responses appears to be important, such as chronic hepatitis B virus (HBV), hepatitis C virus (HCV), and HIV infections and cancer (4–9). The apparent reason for this limitation is likely to hinge on the basic biology of Ag processing and presentation. In general, CTL are efficiently induced when Ag is endogenously synthesized and presented in the context of nascent class I molecules, while HTL are induced by Ags acquired exogenously via endocytosis, phagocytosis, and/or receptor-mediated uptake and presented via the class II receptor (10–13).

Exceptions to this general rule exist, and exogenous Ags can, under certain conditions, access the class I processing and presentation pathway (14–21). In most cases, exogenous Ags that effectively induce class I-restricted responses are particulate, and may access a specialized uptake mechanism by professional APCs (17–21). Class I-restricted presentation can also occur via binding to surface MHC by short peptides generated or present extracellularly (22, 23). Indeed, CTL can be efficiently induced by the use of small "optimal" peptides emulsified in oil-based adjuvants and by lipidated constructs encompassing at least one CTL and one HTL epitope (24).

Multiple CTL and HTL epitopes can be delivered using a single vaccine construct referred to as oligoepitopes or polyepitopes (25–28), minigenes (29, 30) or EpiGenes (31). These experiments were based on genetic constructs delivered either by viral vectors or naked DNA immunization. An advantage of multiepitope vaccines includes the selection of appropriate epitopes which may focus the immune response on conserved epitopes from pathogens commonly characterized by high sequence variability, such as HIV, HBV, HCV and malaria (32–35). Furthermore, epitopes selected from many gene products would increase the breadth of the immune response. Epitopes also may be manipulated to increase potency by altering MHC binding affinity and/or TCR contact residues (36, 37). Finally, multiepitope constructs can be optimized in terms of efficiency by which each epitope is generated in the course of cellular processing (38).

Large polypeptides have been synthesized or produced by recombinant expression technologies (25, 39–41), but their use as vaccines has resulted in generally only Ab and HTL responses (28, 39, 40). The use of adjuvants and multiple immunizations were generally required for high levels of immunogenicity (26, 28, 39, 41).

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[3] Abbreviations used in this paper: HTL, Th lymphocyte; HBV, hepatitis B virus; HCV, hepatitis C virus; PADRE, pan DR epitope; pol, polymerase; SU, IFN-γ secretory unit; SI, stimulation index; DC, dendritic cell; env, envelope; ProGP, Progenipoietin.

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In the present study, a multiepitope polypeptide was evaluated as a means for delivery of both CTL and HTL epitopes. A polypeptide containing six A2-restricted peptides, three A11-restricted peptides, and pan DR epitope (PADRE), a universal HTL epitope (36), was synthesized chemically. Following a single immunization of HLA transgenic mice with the polypeptide in PBS, direct ex vivo and in vitro recall $CD8^+$ and $CD4^+$ responses were evaluated. These responses were compared with those obtained using whole proteins, epitopes in IFA, and naked DNA vaccines. The data obtained support our belief that both CTL and HTL epitopes can be delivered effectively using a multiepitope polypeptide format.

Materials and Methods

Mice

HLA-A2.1/K^b and HLA-A11/K^b transgenic mice used in this study were the F_1 generation derived by crossing homozygous transgenic mice (b haplotype) expressing a chimeric gene consisting of the α1, α2 domains of HLA and the α3 domain of H-2K^b (HLA-A2/K^b; Refs. 42 and 43) with BALB/c mice (The Jackson Laboratory, Bar Harbor, ME). Transgenic mice were bred and maintained at the Epimmune facility (San Diego, CA) following National Institutes of Health guidelines and Institutional Animal Care and Use Committee approved animal protocols.

Cell lines

Target cells used for peptide-specific IFN-γ release and cytotoxicity assays were Jurkat cells transfected with the HLA-A2.1/K^b chimeric gene (42) and 3A4-721.221 tumor cells (44) transfected with HLA-A11/K^b. All cell lines were grown in RPMI complete culture medium, called "R10" (RPMI 1640 medium, 25 mM HEPES, pH 7.4 (Life Technologies, Grand Island, NY), supplemented with 10% FBS, 4 mM L-glutamine, 5×10^{-5} M 2-ME, 0.5 mM sodium pyruvate, 0.1 mM MEM nonessential amino acids, 100 μg/ml streptomycin, and 100 U/ml penicillin).

Immunogens

Peptides

Peptides were synthesized by solid-phase method using Fmoc strategy with 2-(1H-benzotrizole-l-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HBTU) activation (Fastmoc, 0.1-mmol cycles) using an automatic peptide synthesizer (model 433A; Applied Biosystems, Foster City, CA). Chemicals were purchased from Applied Biosystems. The side chain protecting groups used were Trt for asparagine, glutamine, cysteine, and histidine; tBu for serine, threonine, and tyrosine; Boc for lysine and tryptophan; OtBu for aspartic acid and glutamic acid; and Pmc for arginine.

Cleavage of peptide-resin (0.4 g) was completed with a mixture of 9 ml of triflouroacetic acid, 0.5 g of phenol, 0.5 ml of water, 0.5 ml of thioanisole, and 0.5 ml of 1,2 ethanedithiol at 25°C for 6 h. Peptides were precipitated in ice-cold ethyl ether, and washed with ethyl ether, dissolved in DMSO and purified to > 90% by reverse phase HPLC on a 22 × 250 mm Vydac 300A 10-μm C4 column or a Vydac 10-μm reverse phase polymer column (Hesperia, CA). Purity and sequence of the decaepitope peptide was verified by Edman degradation using an Applied Biosystems 492 cLC Protein Sequencer, electrospray mass spectroscopy analysis, amino acid composition analysis, and analytical reverse phase HPLC. The decaepitope polypeptide was observed to be a single peak by reverse phase HPLC and the mass was 11,235 as determined by electrospray mass spectroscopy analysis. Purity of the truncated and monoepitope peptides was also analyzed by electrospray mass spectroscopy analysis and analytical reverse phase HPLC.

Multiepitope peptides were dissolved in DMSO at a concentration of 10–20 mg/ml and stored at −20°C until use. The order and sequence of A2.1-restricted peptides contained within pMin.1 polypeptide are shown in Table I and Fig. 1.

DNA construct

The positions of the epitopes in the DNA EpiGene construct are identical to epitope positions in the decaepitope peptide. The epitopes were selectively positioned to minimize junctional HLA-A2.1, HLA-A11, H-2K^b, and H-2D^b epitopes. The construction of the EpiGene was accomplished by synthesizing eight overlapping oligonucleotides (oligos), averaging ~70 nucleotides in length with 15 nucleotide overlaps (31).

Analysis of peptide solubility

All peptides were prepared as for use in immunization. Briefly, peptide stocks in DMSO were heated to 45°C and diluted into room temperature PBS followed by brief vortexing, to give a concentration of 89 μM. Samples were centrifuged at 50,000 rpm at room temperature for 1 h using a Beckman TL-100 (Fullerton, CA) equipped with a TLA 100.3 rotor. Supernatants were removed and analyzed by measuring absorbance at 280 nm and concentrations were determined based on standard estimated extinction coefficients. Supernatant concentrations were also determined by the Micro BCA assay (Pierce, Rockford, IL). The percentage solubility of the samples was calculated by dividing the measured supernatant concentration by the total starting concentration. Data is presented as the percent solubility, microgram per milliliter solubility, or micromole solubility ± SE.

Immunizations

For polypeptide immunization, immunogens (10–20 mg/ml in DMSO) stored at −20°C were thawed for 10 min at 45°C before being diluted 1/10 (v/v) with room temperature PBS followed by additional 10-fold serial dilutions in PBS. Groups of three to five mice were injected s.c. at the tail base with 100 μl of the dilutions (final, 8.9 and 0.89 nmol/mouse). For the EpiGene vaccine, groups of three to five mice were pretreated for DNA EpiGene immunization by injecting 50 μl of 10 μM cardiotoxin (C9759; Sigma-Aldrich, St. Louis, MO) bilaterally into each tibialis anterior muscle. Five days later, the same muscles received 50 μg of DNA diluted in PBS, for a total of 100 μg/mouse.

Recombinant hepatitis B core protein produced in *Pichia pastoris* (Viral Therapeutics, Ithaca, NY) and full length heterodimeric rHIV-1 IIIb polymerase (pol) 66 produced in an *Escherichia coli* expression system (ImmunoDiagnostics, Woburn, MA) were used for whole protein immunizations. The purity of these proteins was determined by SDS-PAGE electrophoresis. Groups of three to five mice were immunized s.c. at the tail base with 100 μl of the diluted proteins in PBS (2.2 and 0.89 nmol).

For peptides in IFA immunization, frozen peptide aliquots (20 mg/ml) were thawed and diluted in PBS at room temperature, vortexed, and emulsified with an equal volume of IFA (Difco, Detroit, MI) for 20 min in a 5100 Spexmixer/mill (Spex Industries, Metchen, NJ). Groups of three to five mice were immunized s.c. at the tail base with a pool of peptides (8.9 and 0.89 nmol each).

Table I. *Epitopes in the decaepitope polypeptide*

Epitope	Sequence	MHC Restriction	MHC Binding Affinity (IC_{50} nM)[a]	Reference
HBVpol149	HTLWKAGILYK	A11	14	45
PADRE	AKFVAAWTLKAAA	IA[b]	44[b]	36
HBVcore18	FLPSDFFPSV	A2.1	3	46
HIVenv120	KLTPLCVTL	A2.1	102	47
HBVpol551-V	YMDDVVLGV	A2.1	5	A. Sette, unpublished
HBVpol455	GLSRYVARL	A2.1	76	48
HIVpol476	ILKEPVHGV	A2.1	192	49
HBVcore141	STLPETTVVRR	A11	4	45
HIVenv49	TVYYGVPVWK	A11	4	50
HBVenv335	WLSLLVPFV	A2.1	5	51

[a] Binding affinity values presented previously and shown here only for informational purposes.
[b] IA[b] binding affinity is shown. DR binding affinities are given in Ref. 36.

Eleven days to 6 wk following immunization, the mice were sacrificed and the splenocytes were purified for in vitro peptide stimulation or used directly for direct ex vivo ELISPOT measurements. We have done kinetic experiments using these HLA transgenic mice and various immunogens including peptides in IFA, naked DNA, and polypeptides (data not shown). The direct ex vivo T cell responses were generally found to peak from 11 to 14 days. Therefore, we typically use this time point for spleen removal and assay set up.

Assays for IFN-γ release

Responses in immunized animals were measured as a function of IFN-γ production using spleen cells and two related assays, ELISPOT and in situ ELISA (in vitro peptide recall). "In situ" refers to quantitation of IFN-γ using immune cells and does not imply "in situ" IFN-γ measured from histological sections. In the ELISPOT assay, splenocytes were tested for cytokine release following an overnight (18–20 h) peptide (10 μg/ml) activation step. For the in situ ELISA, splenocytes were stimulated in vitro for 6 days with 1 μg/ml of the appropriate A2- and A11-restricted epitopes in the form of peptides. The term "recall," when used in the context of ELISA, is referring to "in vitro peptide recall" responses.

ELISPOT assay (IFN-γ)/CD8$^+$

The $CD8^+$ ELISPOT assay was performed as described (52). The experimental values were expressed as the mean net spots/10^6 $CD8^+$ lymphocytes ± SEM for each peptide. Responses against irrelevant peptides (HBVenv378, sequence, LLPIFFCLWV for A2.1 and MAGE3 69, sequence, SSLPTTMNY for A11) were measured to establish background values that were subtracted from the experimental values. To determine the level of significance, a Student *t* test was performed where $p \leq 0.05$ using the mean of triplicate values of immunized mice vs naive (nonimmunized) mice.

In situ ELISA (IFN-γ)/CD8$^+$

The in situ ELISA (in vitro recall) was performed as described (53). Levels of IFN-γ release were expressed as secretory units (SU). Jurkat-A2.1/K^b and 3A4-721.221-A11/K^b cells were used as APCs. The SU calculation is based on the number of cells that secrete 100 pg of IFN-γ in response to a specific peptide, corrected for the background amount of IFN-γ in the absence of the peptide (53).

^{51}Cr release assay

Six days after peptide restimulation in vitro, the lytic activity of each culture was measured by incubating varying numbers of CTLs with 1×10^4 ^{51}Cr-labeled target cells (Jurkat-A2.1/K^b and 3A4-721.221-A11/K^b) in the presence or absence of peptide in a standard 6-h ^{51}Cr release assay (43). Maximum E:T ratios were routinely between 30 and 40:1.

Measurement of PADRE-specific HTL responses/CD4$^+$

PADRE-specific responses were measured by either [^{3}H]thymidine incorporation in lymphoproliferation assays or IFN-γ production in ELISPOT assays.

Lymphoproliferation assay/CD4$^+$

$CD4^+$ T cells, purified from immunized animals by treatment of splenocytes with anti-L3T4 Ab-coupled beads and separation of cells through a magnetic field (Dynabeads; Dynal Biotech, Oslo, Norway), were cultured in triplicate wells in a flat-bottom 96-well microplate at 2×10^5/well in the presence or absence of 20-fold dilutions of PADRE (20 μg/ml to 6.3 pg/ml). Gamma-irradiated naive splenocytes (RBC lysed) were also added to each well at 5×10^5 cells/well as a source of APC. After 72 h of culture, each well received 1 μCi of [^{3}H]thymidine (ICN Pharmaceuticals, Irvine, CA). Eighteen to 20 h later, the cells were harvested onto a unifilter 96-well plate using a Filtermate Harvester (Packard Instrument, Meriden, CT). After drying, 30 μl of Microscint 20 solution (Packard Instrument) was added to each well and the filter plate was sealed and counted in a Top Counter (Packard Instrument). Lymphoproliferation responses are expressed as a stimulation index (SI) which is calculated as follows: SI = (cpm [^{3}H]thymidine incorporation in the presence of PADRE)/(cpm [^{3}H]thymidine incorporation in the absence of PADRE).

ELISPOT assay (IFN-γ)/CD4$^+$

Briefly, membrane-backed 96-well ELISA plates (Millipore, Bedford, MA) were coated with 50 μl/well of 10 μg/ml anti-mouse IFN-γ (mAb, clone R4-6A2; BD PharMingen, San Diego, CA) and incubated overnight at 4°C. The plates were then blocked using 10% FCS R10 medium and incubated for 1 h at 37°C. Upon removal of blocking medium, 4×10^5 $CD4^+$ splenocytes from immunized animals were added per well to the plates following isolation using MACS Microbeads CD4 (L3T4; Miltenyi Biotec, Auburn, CA) together with peptide (10 μg/ml final concentration) and irradiated (4400 rad) syngeneic splenocytes (1×10^5/well). Plates were then incubated for 20 h at 37°C, 5% CO_2. Plates were washed with PBS/0.05% Tween and wells were incubated with 2 μg/ml biotinylated anti-mouse IFN-γ mAb (clone XMG1.2; BD PharMingen) for 4 h at 37°C. After additional washing, spots were developed by sequential incubation with Avidin-Peroxidase Complex (Vectastain) and AEC (3-amino-9-ethyl-carbazole; Sigma-Aldrich). Spots were counted using the Zeiss KS ELISPOT reader (Graham, WA). The experimental values were expressed as the mean net spots/10^6 $CD4^+$ lymphocytes ± SEM for each peptide. Responses against an irrelevant peptide (HCVcore28, sequence, GQIVGGVYLLPRRGPR) were measured to establish background values that were subtracted from the experimental values. To determine the level of significance, a Student *t* test was performed where $p \leq 0.05$. This Student's *t* test compared the net mean of triplicate values of immunized mice vs the net mean of triplicate values of responses obtained using mice in the naive (nonimmunized) group.

Mobilization and purification of dendritic cells (DCs) by Progenipoietin (ProGP)

ProGP is a member of a family of engineered chimeric proteins that were designed to promote the expansion and differentiation of multilineage hemopoietic progenitor and DCs (54). Mice received ProGP (40 μg/mouse/day) s.c. for 7 days. They were sacrificed and the splenocytes were purified using standard conditions. DCs were affinity purified (55). Recovery was between 4 and 10% of input cells and these cells were ~60% CD11c-positive. The capacity of these DCs to present Ag to A2-specific T cell lines was examined by IFN-γ production using in situ ELISA.

Paraformaldehyde fixation of DCs

DCs were fixed in 0.5% paraformaldehyde (Sigma-Aldrich) at a concentration of 5×10^6 cells/ml in HBSS for 20 min at room temperature. Cells were then washed twice in R10 and used in the in vitro Ag presentation assays.

In vitro Ag presentation assay

CTL lines were generated following immunization using either EpiGenes (31) or lipidated HTL-CTL constructs (24). To maximize the epitope avidity of the CTL lines, low concentrations of peptide were used for in vitro stimulation (0.1–1 μg/ml). DCs were suspended at 5×10^6 cells/ml in OptiMEM-1 (Life Technologies) and pulsed with the indicated concentrations of peptide for 4 h at 37°C. Alternatively, DCs were pulsed with peptide for 4 h at room temp in the presence of 3 μg/ml of β_2m (Scripps Laboratories, La Jolla, CA). Following peptide loading, cells were washed twice in R10 and plated in 96-well plates (Costar, Cambridge, MA) at 1×10^5 cells/well. In these same plates, HBVcore18-specific and HIVpol476-specific CTL lines were added at 1×10^5 cells/well. Plates were incubated

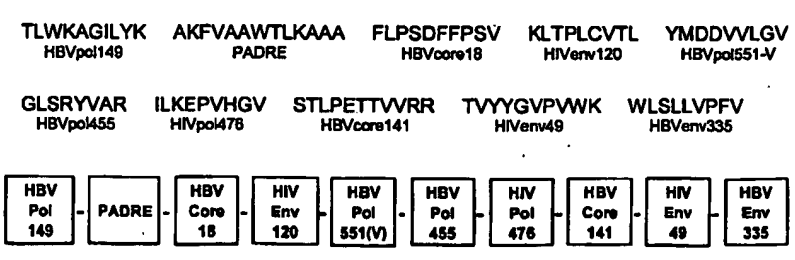

FIGURE 1. Amino acid sequence and position of epitopes within the decaepitope peptide.

overnight at 37°C and tested for IFN-γ release by ELISA as described above. IFN-γ is expressed as picograms per well as determined using IFN-γ standards (BD PharMingen).

Results

Selection of epitopes and decaepitope polypeptide design

We previously described pMin.1, a multiepitope gene (EpiGene) construct encoding various HBV- and HIV-derived CTL epitopes, restricted by the *HLA-A*0201* and *HLA-A*1101* alleles (31). The pMin.1 construct also incorporated the PADRE universal HTL epitope to assist in CTL induction, and an Ig κ-chain derived leader sequence. Responses against all nine CTL epitopes and PADRE were detected following immunization of HLA transgenic mice. In the current study, we evaluated $CD8^+$ IFN-γ and HTL responses induced by a synthetic polypeptide, corresponding in sequence to the polypeptide encoded by the pMin.1 gene. The same sequence was maintained, with regard to both epitope sequence and order. However, for ease of synthesis, a leader sequence was not included in the synthetic polypeptide version. Table I lists the epitopes included in the synthetic decaepitope peptide, and a diagram illustrating its overall structure is presented in Fig. 1. This polypeptide was synthesized and an analytical HPLC profile and a mass spectrometric analysis of the purified product indicated a predominant molecular species of the correct mass (data not shown).

Immunogenicity of the decaepitope polypeptide in HLA transgenic mice

To address the capacity of the polypeptide to prime for IFN-γ responses in vivo, HLA-A2.1/K^b-H-$2^{b \times d}$ and A11/K^b-H-$2^{b \times d}$ transgenic mice were immunized s.c. with 100 μg (8.9 nmol) of this construct dissolved in PBS. HLA transgenic mice were also immunized with a pool of equimolar amounts (8.9 nmol) of each individual epitope dissolved in PBS. As shown in Fig. 2*A*, the decaepitope polypeptide primed for significant IFN-γ in vitro recall responses specific for all nine CTL epitopes, with magnitudes in the 15–1,000 IFN-γ SU range. In contrast, minimal IFN-γ responses were observed when the pool of the nine CTL epitopes and PADRE were used as the immunogen. Finally, using the same conditions, no significant IFN-γ primary in vitro responses were detected using splenocytes from naive mice stimulated in vitro with each of the nine CTL epitopes (2.0 IFN-γ SU or less; Fig. 2*A*). We next wanted to verify whether splenocytes from HLA transgenic mice immunized with the decaepitope peptide were cytotoxic. Following peptide stimulation in vitro, these cells were capable of lysing peptide-coated target cells. As shown in Fig. 2*B*, significant lysis was observed specific for seven of nine peptides in the range of 15–50% ^{51}Cr release. Minimal lysis was observed specific for HBVcore141 and HBVpol149 epitopes in the range of 5–10% ^{51}Cr release. As expected, no significant lysis was demonstrated using splenocytes obtained from animals immunized with an epitope pool in PBS.

Proliferative responses to the PADRE epitope were also measured using splenocytes from the same experimental groups. PADRE-specific proliferative responses with a SI of ~4 were observed following immunization with 100 μg of the decaepitope peptide, but no PADRE-specific proliferation was observed for the peptide pool (SI <2; Fig. 2*C*).

HLA-A2.1/K^b-H-$2^{b \times d}$ transgenic mice were immunized with a dose titration of the polypeptide dissolved in PBS. As shown in Fig. 3*A*, a linear relationship between immunogen dose and A2.1-restricted IFN-γ in vitro recall responses was evident, at least in the 0.089–8.9 nmol dose range (1–100 μg). Significant PADRE-specific HTL responses were also observed at each immunizing dose, in the 2–6 SI range (Fig. 3*B*). These studies demonstrated that the polypeptide can induce IFN-γ, cytotoxicity, and HTL immune responses in vivo, and supported the use of immunogen doses in the 10–100 μg range (0.89–8.9 nmol) for subsequent studies.

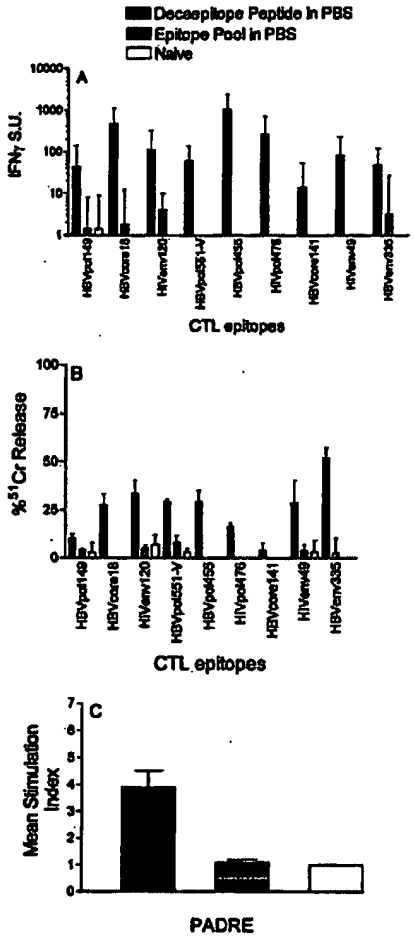

FIGURE 2. $CD8^+$- (IFN-γ), CTL-, and PADRE-specific HTL proliferation responses specific for epitopes within the decaepitope peptide and monoepitope pool. HLA transgenic mice were immunized with the immunogens (8.9 nmoles), 100 μg/mouse of the decaepitope, and 10 μg/mouse of individual epitopes for the pool. Eleven to 14 days after immunization, splenocytes from primed animals were cultured in triplicate flasks and stimulated once in vitro with the appropriate A2- and A11-restricted epitopes. An IFN-γ SU measurement from each flask was determined using an IFN-γ in vitro recall ELISA (*A*). Peptide-loaded Jurkat-A2.1/K^b and 3A4-721.221-A11/K^b were used as APCs (*A*) and target cells (*B*). *B*, Maximum ^{51}Cr release responses were obtained using E:T ratios of between 30 and 40. *C*, Purified $CD4^+$ T cells from immunized animals were cultured in triplicate wells in the presence or absence of varying concentrations of PADRE. Lymphoproliferation responses are expressed as a mean SI. Data presented in *A* are an average of two independent experiments. Data presented in *B* and *C* are a representative example from two independent experiments.

The immunogenicity of the decaepitope polypeptide is not due to contaminating peptides

We addressed the possibility that the immunogenicity of the polypeptide was due to contaminating free monoepitope peptides by using paraformaldehyde-fixed DCs to prevent Ag internalization and processing of Ag. The release of IFN-γ from T cell lines specific for the HBVcore18 and HIVpol476 epitopes was used as

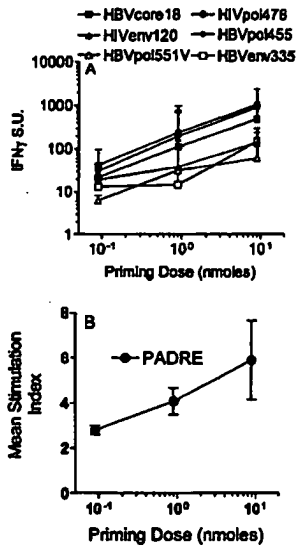

FIGURE 3. $CD8^+$- (IFN-γ) and PADRE-specific proliferation responses specific for epitopes within the decaepitope peptide and monoepitope pool. HLA transgenic mice were immunized with the indicated doses of immunogens in PBS. The assays were performed as described in Fig. 2 and *Materials and Methods*. Data presented are a representative example from two independent experiments.

a functional readout. As shown in Fig. 4, these T cell lines were responsive to peptide-pulsed fixed DCs down to 0.1 ng/ml (HBVcore18-specific) and 10 ng/ml (HIVpol476-specific). By contrast, fixed DCs pulsed up to 100 μg/ml of the polypeptide did not induce any detectable IFN-γ release. These data demonstrate that immunogenicity of the polypeptide was not due to contaminating shorter optimal epitopes, and that Ag processing was required to achieve presentation of the various epitopes contained within the decaepitope construct.

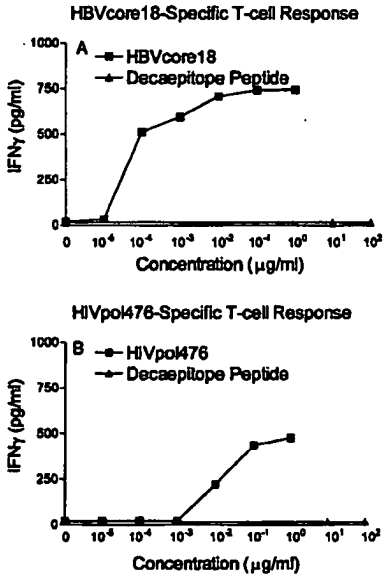

FIGURE 4. In vivo IFN-γ responses are not due to contaminating peptides. In vitro Ag presentation assays were performed using ProGP-mobilized murine DC that were fixed with paraformaldehyde. These APCs were pulsed with either the indicated concentrations of peptides or the decaepitope peptide in the absence of serum. HBVcore18-specific (*A*) and HIVpol476-specific (*B*) cell lines were added to the Ag-pulsed APCs. IFN-γ release was measured by ELISA. Data presented are a representative example of three independent experiments.

Relation between size (number of epitopes) and immunogenicity

The relationship between the size of various multiepitope polypeptides and their immunogenicity was examined using the truncated constructs (Fig. 5*A*). In general, the HBVpol149 and PADRE epitopes were retained at the N terminus and varying numbers of epitopes (1, 2, or 4) were added from the C terminus, thereby retaining the same epitope order and configuration as present in the decaepitope construct. In addition, two constructs containing HBVpol149 and PADRE, or PADRE and HBVenv335, were also synthesized. This design allowed measurement of HBVpol149- and HBVenv335-specific IFN-γ responses induced by these deca-, hexa-, tetra-, tri-, and biepitope constructs as well as Th responses against PADRE.

As shown in Fig. 5*B*, each of the deca-, hexa-, tetra-, tri-, and biepitope constructs induced significant HBVpol149- and HBVenv335-specific IFN-γ in vitro recall responses, in the 40–300 SU range, at the 8.9-nmol immunizing dose. Immunization with the 0.89 nmol yielded HBVpol149-specific IFN-γ responses for the deca- and hexapeptide of 40 and 70 SU, respectively, but no significant responses in the case of the tetra-, tri-, or biepitope constructs. Similarly, in the case of the HBVenv335 epitope, IFN-γ responses in the 10–30 SU range were induced by the lower immunizing dose of the deca-, hexa- and tetraepitope peptides, while no responses were induced by the tri- or biepitope constructs. As expected, control immunizations consisting of either the HBVpol149 or HBVenv335 epitopes failed to induce IFN-γ in vitro recall responses. These results suggested that a threshold size of three to four epitopes (30–40 residues) was associated with priming for optimal $CD8^+$ IFN-γ responses when the lower priming dose of 0.89 nmol was used. However, the higher immunizing dose of 8.9 nmol primed for significant responses even when the immunogen size was only two epitopes. These results suggest that the optimal size for priming of significant responses is dose-dependent.

Immunogenicity of decaepitope polypeptide vs whole proteins and correlation with solubility

The correlation between the solubility of the multiepitope polypeptides and its immunogenicities is summarized in Table II. The deca-, hexa-, tetra-, and triepitope constructs were almost completely insoluble (particulate) with overall percent solubilities of <2%. The biepitope constructs were more soluble, with percent solubilities in the 8–16% range. Lastly, as expected, the A2- and A11-restricted monoepitopes were largely soluble, with a range of 65–96% solubilities measured. Based on these results, solubility, and potentially the presence of particulates, appeared to correlate with immunogenicity.

As a test of the hypothesis that the particulate nature of the multiepitope constructs may be important for induction of $CD8^+$ IFN-γ responses, the immunogenicity of whole proteins was compared with the decaepitope polypeptide. Two proteins, one prone to particle formation (HBV core) and one soluble in aqueous buffers (HIV pol), were chosen for this analysis because they contain epitopes in the decaepitope polypeptide. Direct solubility measurements on these proteins were not performed. As shown in Fig. 6, when the HBVcore18 epitope was delivered by either the polypeptide or whole native Ag, comparable IFN-γ in vitro recall responses in the 32–82 SU range were induced. In contrast, the HIVpol476 epitope induced significant responses only following immunization with the polypeptide (57 and 214 SU at the 0.89 and 2.2 nmol doses, respectively). These results are consistent with our

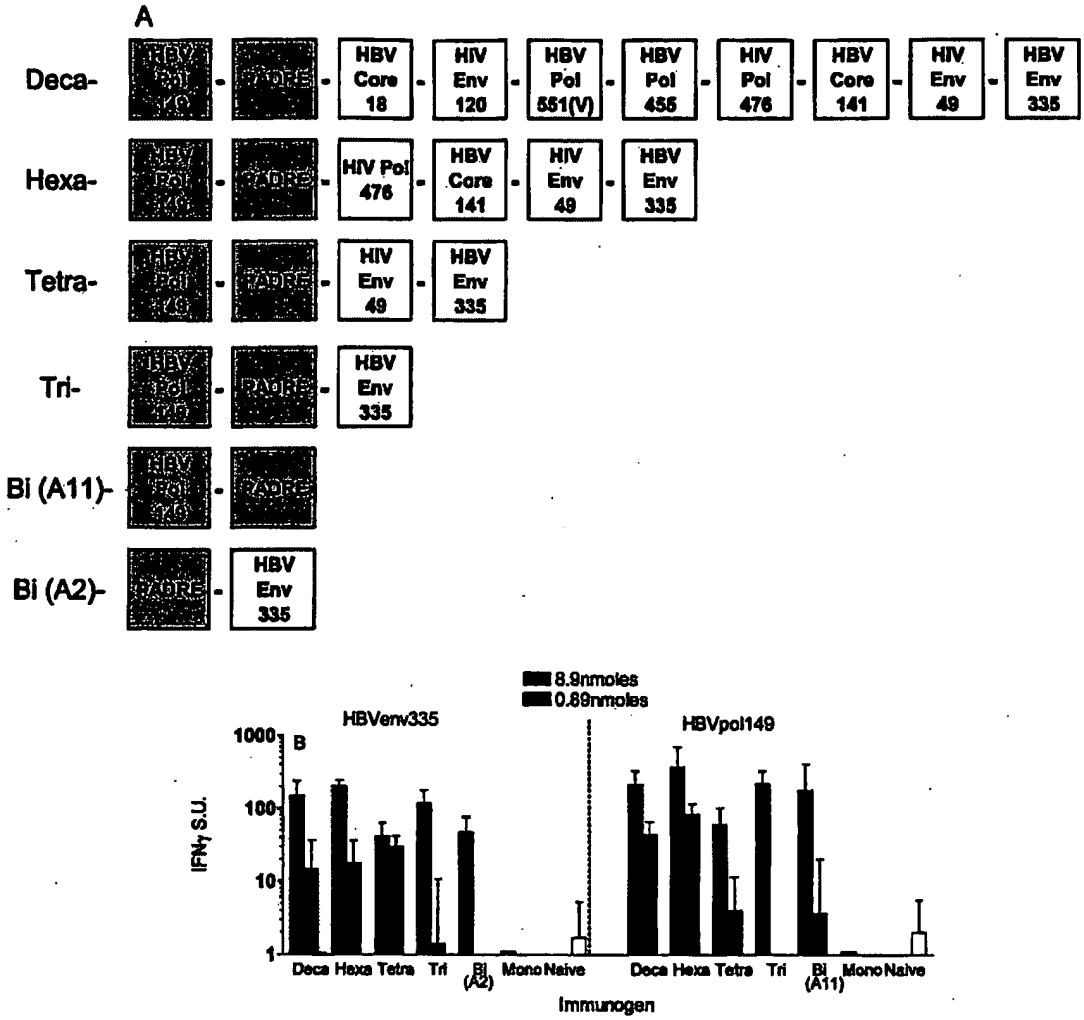

FIGURE 5. Immunogenicity as a function of construct size. HLA transgenic mice were immunized with the decaepitope peptide and various truncations thereof depicted in *A*. *B*, Activated splenocytes obtained 2 wk postimmunization were subsequently analyzed for IFN-γ in vitro recall responses specific for HBVpol149 and HBVenv335. Data presented are an average of two independent experiments.

belief that particulate forms of the Ag are important for effective priming of class I-restricted T cells by exogenous Ags.

Immunogenicity of the decaepitope polypeptide in PBS compared with peptides emulsified in IFA

Immunogenicity of the polypeptide resuspended in PBS was compared with that of a pool of peptide epitopes emulsified in IFA. Both direct ex vivo responses (IFN-γ ELISPOT from fresh splenocytes) and in vitro recall responses (IFN-γ ELISA after one in vitro peptide stimulation) were investigated. As shown in Fig. 7*A*, both the polypeptide in PBS and the peptides in IFA induced IFN-γ direct ex vivo responses in the range of 15–100 spots/10^6 cells shown for three epitopes, HBVcore 18, HBVpol455, and HBVenv335. Only immunization with the peptide pool induced direct ex vivo responses specific for HIVenv120 and neither immunization strategy induced direct ex vivo responses in the case of the HBVpol551V and HIVpol476 epitopes. A difference was observed after the in vitro Ag restimulation where the responses induced by the polypeptide were ~10-fold higher than the epitope pool in IFA (Fig. 7*B*). Thus, the polypeptide may be more potent for priming for memory responses. Similar results were seen when PADRE-specific HTL responses were measured. The peptide pool was superior to the polypeptide in terms of induction of PADRE-specific direct ex vivo responses using freshly isolated splenocytes, 50 vs 15 net spots/10^6 cells, respectively (Fig. 7*C*). However, the polypeptide primed for somewhat higher in vitro recall responses

Table II. *Solubilities of the decaepitope peptide and its derived truncations at 89 μM*

Peptide	% Solubility[a]	μg/ml Solubility[b]	μM Solubility[c]
Deca	0.9 ± 0.4	9.5 ± 3.9	0.8 ± 0.3
Hexa	1.4 ± 0.35	9.2 ± 2.2	1.3 ± 0.3
Tetra	1.6 ± 0.4	7.4 ± 1.9	1.5 ± 0.4
Tri	1.6 ± 0.8	5.5 ± 2.5	1.5 ± 0.7
Bi (A2)	8.3 ± 1.8	19.8 ± 4.2	7.4 ± 1.6
Bi (A11)	16.2 ± 1.2	32.5 ± 3.5	14.4 ± 1.0
Mono (A2)	64.7 ± 2.0	61.8 ± 1.9	57.6 ± 1.7
Mono (A11)	95.8 ± 8.5	113.1 ± 10.1	85.3 ± 7.6

[a] Percent solubility ± SE, values are the average of four values (two for monopeptides) as determined by absorbance at 280 nm and Pierce Micro BCA assay.

[b] Concentration (μg/ml) solubility ± SE; values are the average of four values (two for monopeptides) as determined by absorbance at 280 nm and Pierce Micro BCA assay.

[c] Columns 2 and 3, converted to concentration (μM) solubility ± SE, correspond to the amount of micromolar solubility of a total 89 μM sample (for an 8.9 nmol injection); values are the average of four values (two for monopeptides) as determined by absorbance at 280 nm and Pierce Micro BCA assay.

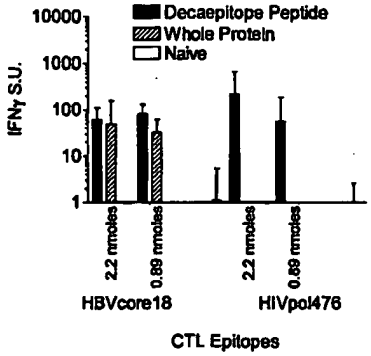

FIGURE 6. Immunogenicity of decaepitope peptide and whole proteins. HLA transgenic mice were immunized with the decaepitope peptide, HBVcore and HIV polymerase whole proteins. Two weeks following immunization, activated splenocytes were used to measure HBVcore18- and HIVpol476-specific IFN-γ in vitro recall responses by ELISA as previously described. Data presented are an average of two independent experiments.

than the epitope pool in IFA, SI of 6 vs 4 (Fig. 7*D*). In conclusion, these data support the previous results which suggested that the polypeptide is more effective in priming for in vitro recall responses, but somewhat less effective in inducing responses of freshly isolated splenocytes.

Induction of IFN-γ responses using decaepitope polypeptide vs naked DNA

Responses induced by immunization with the polypeptide or naked DNA were also compared. Direct ex vivo and in vitro recall IFN-γ responses for four representative A2.1-restricted epitopes were measured 2 and 6 wk after immunization. DNA immunization induced responses for the selected epitopes measured 2 wk post vaccination in the 15–350 spots/10^6 cell range, whereas the polypeptide induced significantly lesser responses, 20 spots/10^6 cells in the case of HBVcore18, and only minimal responses specific for the remaining epitopes (Fig. 8*A*). Both epitope delivery vehicles induced significant IFN-γ responses for each of the four epitopes detected after one in vitro Ag restimulation (Fig. 8*B*). However, DNA immunization was the more potent immunogen, with responses, in general, 5–10-fold higher.

The characteristics of the responses differed when measured 6 wk after immunization. Only minimal direct ex vivo responses were detected in mice immunized with either polypeptide or the DNA immunogens (Fig. 8*C*). However, significant in vitro recall responses, in the 10–300 SU range, were detected only in the group that received the polypeptide immunogen (Fig. 8*D*).

Discussion

In this study, we report that a polypeptide designed to encompass multiple epitopes is an effective immunogen for both $CD8^+$ IFN-γ and HTL responses. Our results suggest that multiepitope polypeptides can be potent immunogens due to their capacity to aggregate and form particulates. Previous studies by other groups have provided similar evidence supporting this hypothesis (17–21). Finally, our results suggest that multiepitope polypeptides might offer significant advantages in terms of priming for memory $CD8^+$ IFN-γ responses, compared with DNA vaccines.

Our study demonstrated that polypeptides can be used to induce potent IFN-γ responses after a single immunization in the absence

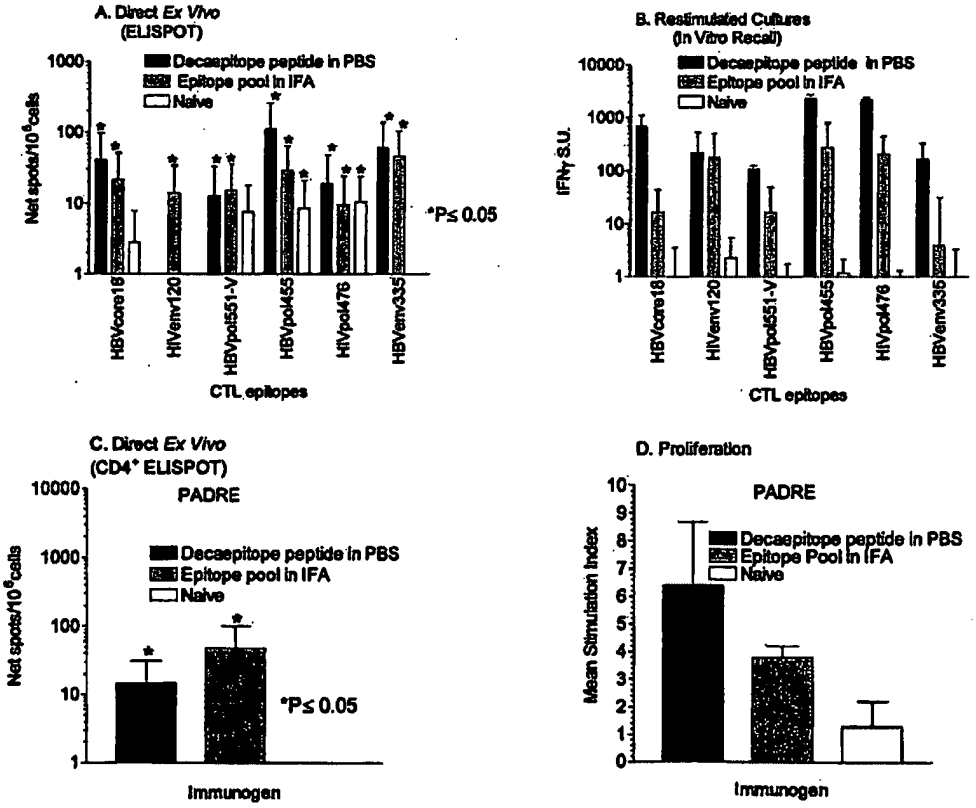

FIGURE 7. Immunogenicity comparison using decaepitope peptide and an epitope pool in IFA. HLA transgenic mice were immunized with the decaepitope peptide (100 μg/mouse) and individual peptides (10 μg of each epitope/mouse) as a pool in IFA. *A* and *B*, Activated splenocytes obtained 2 wk postimmunization were subsequently analyzed for direct ex vivo and in vitro recall A2.1-restricted IFN-γ responses. *C* and *D*, $CD4^+$ PADRE-specific ELISPOT and proliferation responses. Data presented are an average of two independent experiments.

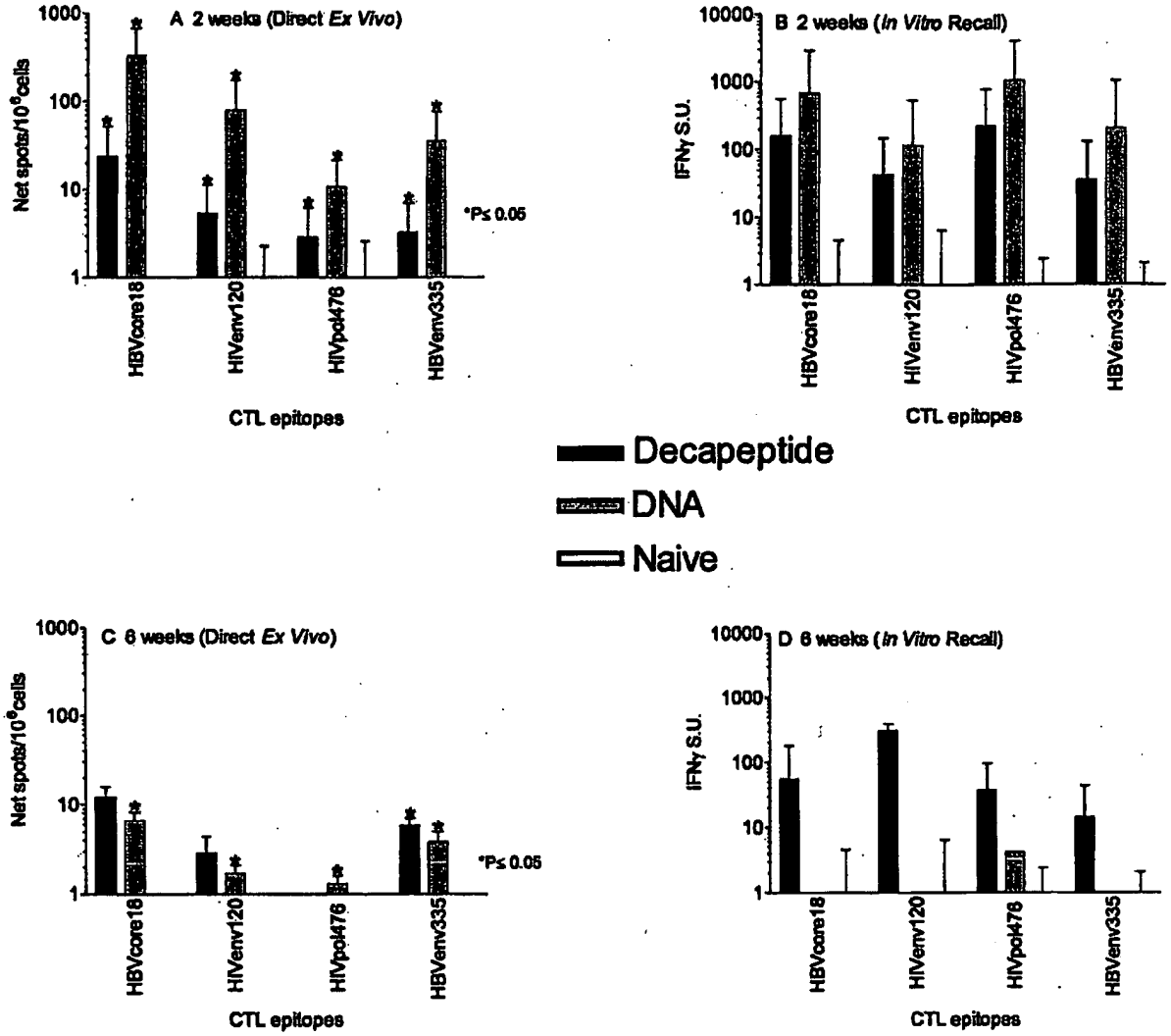

FIGURE 8. Immunogenicity of decaepitope peptide and DNA minigene. HLA transgenic mice were immunized with either 25 μg/mouse decaepitope peptide or 20 μg/mouse naked DNA in the absence of cardiotoxin. Mice were sacrificed at 2 and 6 wk and splenocytes were used for direct ex vivo (*A* and *C*) and in vitro (*B* and *D*) recall immunogenicity measurements. Data presented are an average of two independent experiments.

of any adjuvant. The decaepitope polypeptide described in this study primed for IFN-γ responses specific for all six of the A2- and three of the A11-restricted CTL epitopes tested. In addition, significant PADRE-specific responses were also measured. The induction of CTL responses in HLA-A2.1/K^b transgenic mice following immunization with a 30 residue peptide containing two HLA-A2-restricted epitopes and one HLA-A3-restricted epitope has been described (22).

Our study extends the multiepitope concept to a higher valency (ten epitopes) and increased size (100 residues total). In our case, potent multispecific responses were observed following a single immunization, in absence of any adjuvant and down to a dose of 0.089 nmol, while the multiepitope construct described by Correale et al. (22) seemed to require emulsification in detox adjuvant and a total of three injections. The increased potency of our polypeptide might be at least in part due to peptide size (a 30 residue size in our hands was associated with suboptimal potency) and to the inclusion of a helper $CD4^+$ epitope (24, 56).

The immunogenicity of the polypeptide was not due to contaminating smaller m.w. peptides and Ag processing was required. It is possible that the polypeptide was processed and presented by the classical class I pathway via a cross-priming mechanism (57–59). Alternatively, extracellular processing of the polypeptide could account for loading of class I cell surface molecules (22, 23). The finding that optimal activity is associated with particulate peptide forms is most consistent with peptide uptake by APCs as being the basis for induction of T cell responses. However, direct experimental evidence of particulate uptake by APCs in this system is not available. The fact that shorter, more soluble peptides are associated with lower activity is not immediately suggestive of extracellular processing, as these peptide forms would also be susceptible to extracellular proteolysis.

The immunogenicity of truncated polypeptide constructs correlated with decreased levels of solubility, thus supporting the notion that aggregated decaepitope peptide might be important for effective induction of $CD8^+$ IFN-γ responses. Consistent with this idea, we demonstrated that a soluble protein such as HIV polymerase, was unable to prime for pol476-specific $CD8^+$ IFN-γ responses, while the particulate HBV core protein primed significant core18-specific responses.

In general, the decaepitope polypeptide was most effective in priming for in vitro recall responses to both class I- and II-restricted epitopes than either peptide in IFA or DNA vaccines. The reason for these differences is unclear, but might reflect accessing

multiple APC populations resulting in varying patterns of Ag persistence, thus potentially influencing initial clonal burst size and therefore the quality of the pool of memory cells resulting from immunization (60–62). Taken together, the differential potencies of the polypeptide and DNA may best be exploited using a prime boost protocol. Numerous studies have illustrated the potential benefit of a DNA prime followed by a viral boost for induction of immune responses (63–65). Our results suggest that multiepitope polypeptides could be similarly investigated. Polyvalent synthetic peptide immunogens might in fact offer distinct safety advantages, as compared with use of viral vectors, especially in immunocompromised individuals.

We succeeded in synthetically generating a polypeptide of 100 residues in size by standard automated peptide synthesis. Although other investigators have successfully synthesized long synthetic peptides encompassing *Plasmodium falciparum* proteins (39, 41), this is the first report of synthesis and characterization of a peptide of 100 residues in size, which has been engineered using small epitopes as building blocks. Such constructs are likely to lack defined protein folding patterns and might have a tendency to form aggregates. This feature appears to be related to their poor solubility and high immunogenicity, but might also present an issue for synthetic production. Further improvements in synthetic peptide chemistries may expand the size of polypeptides that are amenable to synthetic production. Potential alternatives include chemoselective ligation of purified intermediates (66). Alternatively, various recombinant expression systems could be used for generating large peptides such as transgenic plants (67), bacteria (40), yeast (68), and baculovirus-insect cells (28, 69).

We used synthetic polypeptides devoid of any specific targeting sequences. However, it is possible that additional gains in potency might be realized by inclusion of specific sequences targeting the immunogen to the cytoplasm of APCs. Interesting alternatives include complexes of tat-like sequences (70, 71) or bacterial toxins (72). Investigation of the potential of these strategies as a means to further improve potency of polypeptide constructs is currently ongoing in our laboratory.

Finally, it will also be important to determine the potency of polypeptide constructs in higher animals, such as nonhuman primates and humans. We are encouraged by a recent study (26) that reported induction of CTL responses in humans after repeated immunization with a multiepitope polypeptide emulsified in IFA. We are also currently designing immunogens suitable for studies in the Rhesus macaque system, taking advantage of recently defined HTL and CTL epitopes for several MHC types commonly expressed in macaques (73, 74).

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